

IN VITRO ANTHELMINTIC EFFECT OF *ANOGEISSUS LEOCARPUS* (DC.) GUILL. & PERR. LEAF EXTRACTS AND FRACTIONS ON DEVELOPMENTAL STAGES OF *HAEMONCHUS CONTORTUS*I. O. Ademola^{1a}, J. N. Eloff^{1*}

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Abstract

The anthelmintic effect of acetone leaf extract and fractions of *Anogeissus leiocarpus* was investigated to determine the relative efficacy of the components as anthelmintic against *Haemonchus contortus* (Rudolphi). The fractions were obtained by solvent-solvent group separation of the leaf extract. The fractions were evaluated for ovicidal and larvicidal activity by egg hatch inhibition assay and larval development viability assay. Best-fit LC₅₀ values for egg hatch test were 0.360, 0.316, 0.093, 0.219 and 0.196 mg/ml for the crude acetone extract, hexane, chloroform, butanol, and 35% water in methanol fractions, respectively. While the best-fit LC₅₀ values for larval development and viability test were 0.509, 0.162, 0.186, 0.288 and 0.130 mg/ml for the crude acetone extract, hexane, chloroform, butanol, and 35% water in methanol fractions, respectively. The 35% water in methanol fractions was the more active on larvae, although differences in activity between fractions were not significant ($p > 0.05$). *A. leiocarpus* leaf extracts could find application in anthelmintic therapy in veterinary practice.

Key words: *Anogeissus leiocarpus*; anthelmintic; *Haemonchus contortus*; eggs; larvae

Introduction

Helminthosis is considered to be a major cause of mortality and sub-optimal productivity in goats and sheep in traditional farming systems in sub-Saharan countries. Helminth cause direct losses due to deaths and indirect losses due to reduced productivity through reduced feed intake and live weight gains and, decreased quality of skins and wool (Soulsby 1982). Furthermore, they render animals more susceptible to other infections. In most countries the use of manufactured anthelmintics is currently seen as the most effective means for their control. But synthetic anthelmintics have some serious disadvantages, such as non-availability in some developing countries, cost and risk of misuse leading to drug resistance, environmental pollution and food residues. According to circumstances and depending on their efficacy, naturally produced plant anthelmintics offer an alternative that can overcome some of these problems and is both sustainable and environmentally acceptable.

Anogeissus leiocarpus (DC) Guill and Perr family Combretaceae (Common name: Axlewood tree) has many applications. Leaves, roots and trunk bark of *A. leiocarpus* are used by traditional practitioners for the treatment of helminthiasis, trypanosomiasis, malaria and dysenteric syndrome. It is also used in traditional medicine as a remedy for many ailments of livestock and man, which include schistosomiasis, leprosy, diarrhoea and psoriasis (Burkill, 1985). Some members of the Combretaceae have high concentrations of flavonoids, terpenoids, tannins or polyphenolic compounds (Eloff et al, 2008).

In general, the influence of herbal medicine and natural products upon drug discovery is impressive and a number of clinically active drugs are either natural products or have a natural product pharmacophore (Koehn and Carter, 2005). The need to study medicinal plants in detail from various points of view, to discover new therapeutically active compounds, or to understand which component of plant is responsible for the activity and validate their toxic effect therefore becomes imperative. The present study therefore investigates the in vitro anthelmintic activity of an acetone extract of *A. leiocarpus* and of fractions obtained by solvent-solvent fractionation.

Materials and Methods

Plant extracts preparation

The leaf of the plant *A. leiocarpus* was collected in Zaria, Nigeria. Voucher specimens (No: 167) were identified and deposited by the herbarium section of the biological sciences department, Ahmadu Bello University, Zaria. The plant material was air dried and ground to powder using a Macsalab Model 200 LAB grinder. The extract was prepared by maceration with shaking 180 g of the powdered plant (Labotec Model 20.2 shaker) for 24 h in 70% acetone with a 10:1 solvent to dry weight ratio

(Eloff, 1998) and the extract was filtered through Whatman No 1 filter paper using a Buchner funnel, and the acetone removed by air drying.

A slight variation of the solvent:solvent group separation procedure used by the USA National Cancer Institute (Suffness and Douros, 1979) was adopted to fractionate the acetone extract with. The acetone extract (16.27 g) was taken to dryness in a rotary evaporator under reduced pressure and this extract was dissolved in a 1:1 mixture of chloroform and water. The water fraction was extracted with an equal volume of butanol in a separating funnel to yield the water and butanol fractions. The chloroform fraction was taken to dryness in a rotary evaporator under reduced pressure and extracted with a 1:1 mixture of hexane and 10% water in methanol. The hexane fraction was recovered with a separating funnel. The 20% water in methanol extract was diluted to 35% methanol in water and extracted with chloroform to yield the chloroform fraction and the 35% water in methanol fractions. In all cases equal volumes of the solvents were used and the extraction was repeated with a small volume approximately three more times or until the entire colour was extracted.

***H. contortus* egg recovery**

H. contortus eggs were recovered according to Hubert and Kerboeuf (1992). A sample of faeces (45 to 50 g) from sheep experimentally infected with mono-specific larval suspensions of fresh *H. contortus*. Faecal culture for third-stage nematode larvae revealed 100% *H. contortus* in this animal. The faecal sample was suspended in water and cleared of organic debris by filtration through 1 mm and 150 µm sieves. Eggs were collected on a 25 µm sieve and further cleared of organic debris by centrifugation in magnesium sulphate (density 1.10 g/cm³) for five minutes at 1000 g. The supernatant was filtered through 100 µm and 63 µm sieves and the eggs were washed in water and collected on a 25 µm sieve. The concentration of eggs was estimated in 200 µl samples and adjusted to 500 eggs/ml. Amphotericin B (5 µg/ml) solution (Sigma) was added to the egg suspension to avoid fungal development.

Egg hatch assay

The in vitro egg hatch assay method described by Coles et al. (1992) was adopted. A suspension of 0.2 ml was distributed in a 48-flat-bottomed microtitre plate containing approximately 100 fresh eggs/well and mixed with the same volume of plant extract at concentrations of 10 mg/ml in 8 serial dilutions. Fractions were tested at a concentration of 1 mg/ml in 8 serial dilutions. Albendazole (99.8% pure standard reference) was used as a positive control. Albendazole was dissolved in dimethyl sulfoxide (DMSO) and diluted at the concentrations of between 1 µg/ml and 0.0075 µg/ml. The control plates contained the diluents water and acetone or 0.3% DMSO, depending on the extract, and the egg solution. The eggs were incubated in this mixture for 48 h at 27 °C and 70% relative humidity. After this time a drop Lugol iodine solution (Reidel de Hae) was added to stop the eggs from hatching. All the eggs and first-stage larvae (L₁) in each plate were counted. There were three replicates for each concentration and control.

Larval development and viability assay

The procedures used were a modification of the technique described by Hubert and Kerboeuf (1992). Aliquots of 150 µl of a suspension with about 100 eggs/well, and 20 µl of filtrate obtained by faecal washing during egg recovering were distributed to wells of a 48-well flat-bottomed microplate. This suspension was supplemented with 30 µl of the nutritive medium described by Hubert and Kerboeuf (1984) and comprised of Earle's balanced salt solution (Sigma) plus yeast extract (Sigma) in saline solution (1 g of yeast extract/90 ml of saline solution) at a ratio of 1:9 v/v. The plates were incubated at 27°C and 70% relative humidity. After 48 h, 200 µl of the extract or diluent (control) were added at concentrations of 10 mg/ml in 8 serial dilutions. Fractions were tested at concentrations of 1 mg/ml in 8 serial dilutions. The third stage larvae were obtained six days later. At this time the parasites were counted by separating the larvae into two classes, third-stage larvae (L₃) and other developmental stages larvae (L₁ and L₂). There were three replicates for each concentration and control. The solvent used to dilute the plant extract and albendazole (acetone and 0.3% DMSO, respectively) did not inhibit egg hatching.

Statistical analysis

The LC₅₀ was determined by computing the concentration of extract that gave a response halfway between the minimum and maximum responses in a concentration-response sigmoid curve. The relation below gives the egg hatch and larval development parameter respectively:

$$\frac{\text{Number of larvae} / \text{Total number of larvae and eggs in wells with plant extracts}}{\text{Number of larvae} / \text{Total number of larvae and eggs in control wells (water)}} \\ \frac{\text{Number of living L3} / \text{Total number of larvae in wells with plant extracts}}{\text{Number of living L3} / \text{Total number of larvae in control wells (water)}}$$

Determination of LC₅₀ of a sigmoidal concentration response (variable slope) curve was performed using GraphPad Prism version 4.01 for Windows (GraphPad, San Diego, CA, USA). The analysis of the family of data sets generated by four

solvent:solvent fractions tested was performed by the global curve-fitting model of nonlinear regression analysis with top and bottom shared among the data sets. In addition, the bottom of the curve was constrained as >0 and the top was constrained as <1.0 . A (global) best-fit value that applies to the family of data sets was computed for each of these shared parameters, while best-fit LC_{50} value (unshared parameter) was calculated with 95% confidence interval for each of the data sets (fractions). The relative bioactivity of the fractions was further assessed by comparing the best-fit LC_{50} value of the various fractions by one-way ANOVA and Tukey's multiple comparison test which was performed using GraphPad Prism version 4.01 for Windows.

Results

Yield of extract and fractions

The acetone extract gave a yield of 19.51 g (10.84%), while the hexane, chloroform, butanol and 35% water in methanol fractions gave a yield of 4.74 g (29.14%), 4.94 g (30.36%), 0.91 g (5.6%) and 0.95 g (5.83%), respectively. The water fraction was excluded from the study due to logistical problems with our freeze dryer therefore it was excluded from the study.

Egg hatch assay

A. leiocarpus acetone extract and the fractions inhibited egg hatching in dose dependent response (Figure 1). The shared statistical parameters of the curve fitting analysis and the best-fit LC_{50} values for the acetone extract and the fractions are shown in Table 1. The best-fit LC_{50} values were calculated with reasonable precision (95% CI). Albendazole inhibited egg hatch of 50% of the eggs at a concentration (0.083 $\mu\text{g/mL}$), indicating susceptibility of the strain of *H. contortus* used in the current study.

Larval development and viability assay

A. leiocarpus extracts inhibited larval development and killed the larvae in a concentration dependent manner (Figure 2). The shared statistical parameters of the curve fitting analysis and the best-fit LC_{50} values for the acetone extract and the fractions are shown in Table 2. The best-fit LC_{50} values were calculated with reasonable precision (95% CI). The 35% water in methanol fraction was the most active of the fractions. Tukey's multiple comparison (post ANOVA) test shows that there was no significant difference in the activity of the fractions on egg hatch inhibition and larval development and viability tests. Albendazole produced LC_{50} at low concentration (0.061 $\mu\text{g/mL}$), indicating susceptibility of the larval development of the strain of *H. contortus* used.

Table 1: Egg hatched assay LC_{50} of *A. leiocarpus* test extract and fractions using global sigmoidal (4-parameter logistic) model of curve-fitting.

Fractions	Log LC_{50}		LC_{50} (mg/mL) ^a		R^2
	Best-fit	Std. Error	Best-fit	95% CI	
A	-0.4440	0.05338	0.3597	0.2784 to 0.4649	0.9824
H	-0.5004	0.1596	0.3160	0.1471 to 0.6786	0.9084
C	-1.031	0.2707	0.09318	0.02548 to 0.3408	0.7194
B	-0.6592	0.1856	0.2192	0.09010 to 0.5332	0.8650
MW	-0.7090	0.2694	0.1955	0.05379 to 0.7102	0.7468
Albendazole ^b	-0.7865	0.09927	0.08310	0.1015 to 0.2634	0.8157

^aGlobal shared parameters for acetone extract and fractions.

^b $\mu\text{g/mL}$ A: Acetone crude extract; C: Chloroform; H: Hexane; B: Butanol; MW: 35% Water in methanol fractions.

Table 2: Larval development and viability assay LC_{50} of *A. leiocarpus* test extract and fractions using global sigmoidal (4-parameter logistic) model of curve-fitting.

Fractions	Log LC_{50}		LC_{50} (mg/mL) ^a		R^2
	Best-fit	Std. Error	Best-fit	95% CI	
A	-0.2937	0.06652	0.5085	0.3695 to 0.7000	0.9844
H	-0.7899	0.1766	0.1622	0.07216 to 0.3646	0.7980
C	-0.7308	0.08088	0.1859	0.1283 to 0.2693	0.8293
B	-0.5400	0.09011	0.2884	0.1908 to 0.4359	0.8473
MW	-0.8877	0.08461	0.1295	0.08787 to 0.1909	0.7308
Albendazole ^b	-0.8422	0.08774	0.0610	0.09436 to 0.2192	0.8076

^aGlobal shared parameters for fractions A-D.

^b $\mu\text{g/mL}$ A: Acetone crude extract; C: Chloroform; H: Hexane; B: Butanol; MW: 35% Water in methanol fractions

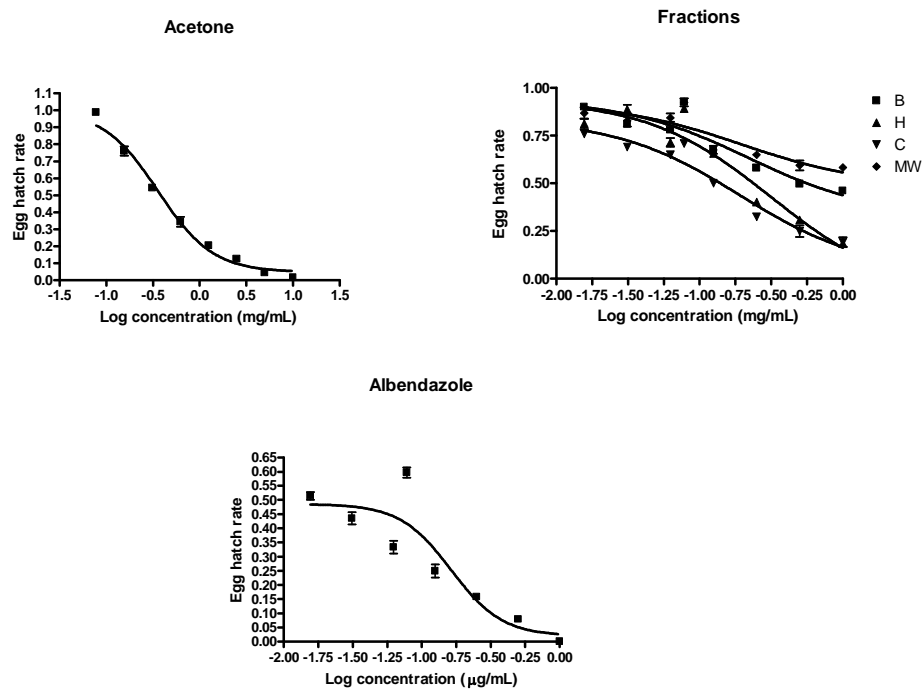


Figure 1: Egg hatch assay concentration–response curve of acetone extract and fractions of *A. leiocarpus*, and Albendazole against eggs of *H. contortus* using global sigmoidal model of curve fitting. (C: Chloroform; H: Hexane; B: Butanol; MW: 35% Water in methanol).

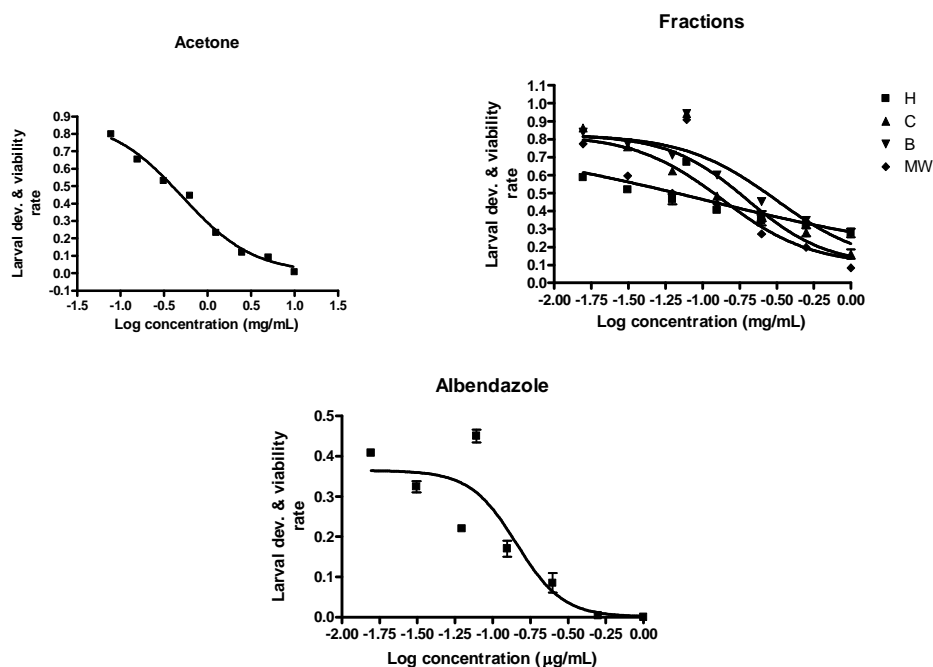


Figure 2: Larval development and viability assay concentration–response curve of acetone extract and fractions of *A. leiocarpus*, and Albendazole against larvae of *H. contortus* using global sigmoidal model of curve fitting. (C: Chloroform; H: Hexane; B: Butanol; MW: 35% Water in methanol).

Discussion

There were no statistically significant differences in the activity of the different fractions. This probably means that more than one compound is responsible for the activity. As far as the egg hatching is concerned the chloroform fraction had the lowest LC₅₀. It therefore appears that relatively non-polar compounds play a role. As far as larval development is concerned more polar compounds may play a role because the 35% water in methanol fraction had the best activity. The more polar (butanol) and less polar (chloroform and hexane fractions) had lower activities. These results are more or less in the line with results found with the anthelmintic activity of *Combretum molle* (Ademola and Eloff, 2010).

Aqueous and hydro-alcoholic extracts of *Hedera helix* had LC₅₀ of 0.12 and 0.17 mg/ml respectively when tested against *H. contortus* egg (Egale et al., 2007a). Aqueous extract of *Coriandrum sativum* had an LC₅₀ of 0.12 mg/ml while the hydro-alcoholic extract had an LC₅₀ of 0.18 mg/ml on egg hatch inhibition test (Egale et al., 2007b). This is comparable to the of *A. leiocarpus* activity observed in this study. However Agaie et al (2007) reported acute toxicity effect of aqueous leaf extract of *A. leiocarpus* in rat.

A. leiocarpus leaves have been reported to contain a variety of compounds. The presence of 3,4,3-tri-*O*-methylflavellagic acid and its glucoside were recently described in this species. This glucoside showed antimicrobial activity on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* (Adigun et al., 2000). The terpenoidal fractions of *A. leiocarpus* had antimicrobial activities against *S. aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Mann et al 2008). *Anogeissus leiocarpus* is effective against adult *Nippostrongylus braziliensis* in rats at non-toxic doses (Ibrahim et al 1983). The chemotherapeutic links between *N. braziliensis* and trichostrongyles in sheep and between *N. braziliensis* and hookworms in dogs and man have been established (Whitlock, 1945; Standen, 1963). Other phytochemicals reported to have anthelmintic effect include essential oils (Pessoa et al., 2002), tannins, flavonoids and terpenoids (Athnasiadou et al., 2001; Lahlou, 2002; Alvi et al, 1991).

The egg hatch inhibition and larval development assays provided evidence that all the fractions affected egg and larva survival and their relative potencies is comparable because the difference in biological response is not statistically significant. The activity by different chemical compounds also suggest multiple mechanisms of action which may be useful for efficacy and prevention of anthelmintic resistance.

Conclusion

A. leiocarpus appears to possess some anthelmintic properties that may support the use of this plant by local farmers in traditional animal health care. The activity of the crude extract and fractions are three orders of magnitude lower than albendazole. Because the extract may have its effect in the gut without having to be taken up in the parenteral system high concentrations may be attainable if there are no toxicity problems. Further *in vitro* and *in vivo* experiments that will incorporate toxicity and toxic residue studies are required before *A. leiocarpus*, or any plant, can be recommended for safe use. Isolation and elucidation of the active principles could provide a lead to a potent anthelmintic.

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